Subject: Resubmission of Personal Research Grant (Application no. 3231/24)

Dear ISF Review Committee,

I would like to express my sincere gratitude to the reviewers for their valuable and constructive feedback on my previous submission. Their insights and suggestions have been instrumental in refining my proposal “Elucidating the Cellular and Molecular Mechanisms of Rootward Na+ Recycling Under Salt Stress Conditions”. I am pleased to resubmit my revised application for your consideration.

This proposed research addresses a critical global challenge- soil salinity- that significantly impacts agricultural productivity. By focusing on the relatively unexplored mechanism of Na+ recycling from shoots toward roots via phloem transport, this project aims to enhance our understanding of plant salt tolerance. The multidisciplinary approach, which includes single-cell transcriptomics, fluorescence imaging, and whole-plant physiological analysis, is designed to identify key genes involved in this process. This work holds potential for practical applications in improving crop salt tolerance and addressing broader environmental challenges.

In this revised version, I have carefully addressed the reviewers' comments, specifically, the three main concerns raised by reviewer #2, thereby enhancing the feasibility and scientific impact of the project:

1. Fluorophore selection for companion cell and phloem parenchyma labeling: The reviewer suggested an alternative to Venus for cell labeling due to potential spectral overlap with CoroNa green. I have addressed this by updating the fluorophore to tdTomato, which has an emission peak (Em λ) at 581 nm, allowing for distinct emission separation when used alongside CoroNa green (Em λ = 516 nm) and the cell wall dye calcofluor white (Em λ = 347 nm). The proposed fluorophore and dye set can be visualized using the Zeiss LSM900 confocal microscope, available at the Bioimaging Unit of the University of Haifa. These adjustments are expected to optimize imaging and quantification of Na+ dynamics, as detailed in the revised Objective 1a.
2. Limitations of CoroNa green for Na+ quantification: The reviewer has accurately identified the limitations associated with the use of CoroNa green. As a single-excitation/single-emission probe, it does not exhibit shifts in fluorescence emission spectra upon binding to Na⁺. Furthermore, its fluorescence intensity is influenced by various factors, including the amount of dye taken up by the cells, the extent of hydrolysis in the cytosol, and photobleaching, all of which can compromise the reliability of steady-state Na⁺ measurements. I acknowledge these limitations and have directly addressed the reviewer’s concerns through the following approaches:
   1. Cross-validation with ICP-MS: I employed inductively coupled plasma mass spectrometry (ICP-MS) in conjunction with fluorescence measurements. This yielded a strong positive correlation (rs=0.75, pV=0.03) in Na⁺ concentration between the two methods, thereby enhancing confidence in the dye-based measurements.
   2. Optimization of dye delivery and imaging protocols: To minimize variability in dye uptake and hydrolysis, I refined the dye loading protocol. This included optimizing the section thickness (testing thicknesses from 100 to 500 µm in both wild and domesticated tomato leaves), adjusting incubation times, assessing cell viability using fluorescein diacetate, and improving imaging acquisition methods.
   3. Quantification of dynamic changes in Na⁺ concentration: Similar to many fluorescence-based methods, analyses are limited to single experiments. I focus on quantifying changes in Na⁺ levels within specific cell types or tissues across sections collected from the same experimental setup rather than making comparisons of steady-state or baseline Na⁺ levels between different experiments. This approach reduces the impact of variability in dye uptake or bleaching while effectively detecting Na⁺ fluctuations.

Utilizing these methodologies, I successfully quantified changes in Na⁺ levels in live leaf cross-sections from up to 60 plants in a single experiment. Furthermore, the enhanced accumulation of Na⁺ in distinct cell populations was consistently observed across independent experiments, both within the same species and among different tomato species.

While alternative methods for quantifying Na⁺ concentrations exist, they also have notable limitations. For instance, CryoSEM coupled with energy-dispersive X-ray spectroscopy (CryoEDX) faces inherent limitations in elemental detection sensitivity, spatial resolution, and potential artifacts from ice crystal formation during sample preparation, which can collectively constrain reliable Na⁺ quantification (McCully *et al*., 2010). Cryogenic Nanoscale Secondary Ion Mass Spectrometry (Cryo-nanoSIMS) provides impressive nanoscale resolution and elemental sensitivity, but it is hindered by sample preparation complexity, spatial resolution constraints, high costs, and limited throughput (Meibom *et al*., 2023).

1. Challenges of plant protoplast sorting: The reviewer has rightly noted the challenges associated with plant protoplast sorting. Plant protoplasts are generally larger than animal cells, necessitating a larger nozzle size (typically 100 µm or more) to prevent clogging and minimize shear stress during sorting. I fully acknowledge the difficulties related to protoplasts' fragility and potential loss of viability during this process, which are aggravated by their size and lack of cell walls.

I have considerable experience in generating a large number of viable protoplasts from *Physcomitrium patens* tissues, particularly from young leafy buds, which present challenges due to their compactness and heterogeneous cell sizes. However, I lack firsthand experience in protoplast sorting and transfection, specifically in tomatoes. To address this gap, I have reached out to Dr. Idan Efroni (HUJI), a recognized expert in both techniques, for guidance and training for my lab members. Dr. Efroni's laboratory routinely employs fluorescence-activated cell sorting of tomato protoplasts (Omary *et al*., 2022; Coleto-Alcudia *et al*., 2024) and has developed a rapid transfection assay for protoplasts (Bargmann *et al*., 2013). His scientific support and extensive experience will be invaluable assets for the successful completion of this work.

**Response to reviewer #1:**

1. Benefits of rootward Na⁺ recycling: The reviewer questions why plants would invest energy in rootward Na⁺ recycling, given that Na⁺ loading into the xylem is an active, controllable process. The primary advantage of rootward Na⁺ recycling lies in its ability to prevent toxic accumulation in photosynthetic tissues, where excess Na⁺ can disrupt essential metabolic processes like photosynthesis. While regulating Na⁺ loading into the xylem is an active process and a common focus in salt tolerance studies, recycling additionally supports ion homeostasis, prevents osmotic stress, and protects the plant’s water balance. Additionally, recycling contributes to maintaining a favorable K⁺/Na⁺ ratio and provides adaptability under fluctuating salt stress by enabling the plant to sequester excess Na⁺ in less sensitive tissues, such as old leaves, stems, or roots (Tester & Devenport, 2003, Berthomieu *et al*., 2003). This strategy extends beyond mere control of Na⁺ uptake, allowing plants to dynamically adapt to salinity conditions, making it a more sustainable long-term approach. These clarifications were integrated into the research proposal.
2. Phloem buffering capacity: The reviewer raises an important question regarding the buffering capacity of the phloem, noting that as a metabolically active tissue, Na⁺ accumulation could interfere with its function. While Na⁺ can accumulate in the phloem under salt stress and disrupt sugar transport, existing literature suggests that this effect is concentration-dependent and influenced by the plant’s ability to compartmentalize excess ions (Wolf, 1990; Alfocea, 2000; Munns & Tester, 2008). The working hypothesis, tested in Objectives 1 and 2, is that Na⁺ accumulation in phloem parenchyma or companion cells is transient and facilitates Na⁺ loading into sieve elements for long-distance transport toward the roots. I propose that the phloem's buffering capacity is modulated by its metabolic activity and the presence of transporters that help maintain ion balance, as demonstrated by the recent finding of enriched K⁺ channel expression in Arabidopsis companion cells (Kim, 2021).

**Additional response to reviewer #2:**

1. Rationale for assessing Δ18O in transpired water: Measuring Δ18O in transpired water allows us to trace water movement within the plant, as the isotope ratio reveals both the source and pathways of water flow. By analyzing Δ18O, we can differentiate between water newly absorbed by the roots and water that is recirculated within the plant, providing a clearer picture of shootward water flow dynamics. This approach offers a non-invasive, precise method to quantify plant water uptake and transport efficiency under both control and salt stress conditions. A more detailed explanation of this rationale has been incorporated into the research proposal.
2. Pharmacological approach for Na+ transport studies: The utility of pharmacological approaches for studying Na+ transport mechanisms *in planta* has been well established through multiple independent studies. Several types of inhibitors have proven particularly valuable, including membrane-permeable cyclic nucleotides (cAMP and cGMP) that effectively inhibit Na+ influx through voltage-independent channels in intact Arabidopsis plants (Maathuis & Sanders 2001). Similarly, the non-specific cation channel blocker quinine has been successfully employed to inhibit root Na+ transport (Essah *et al*., 2003). Additionally, amiloride, a specific inhibitor of Na+/H+ antiporters, has revealed distinct patterns of Na+ accumulation and redistribution in tomato plants, particularly in leaf Na+ recirculation (Taleisnik *et al*., 1991). While these pharmacological approaches present certain technical challenges, they remain valuable tools for dissecting Na+ transport mechanisms *in planta*. By applying these inhibitors across different genetic backgrounds, including mutants with altered Na+ transport pathways, we can systematically evaluate the relative contributions of specific transport mechanisms. This approach provides a robust framework for analyzing rootward Na+ transport, as detailed in the revised research proposal.
3. We appreciate the reviewer's thorough review. The identified error in the budget justification has been corrected.
4. I appreciate the reviewer's attention to feasibility concerns. My laboratory is fully equipped for the proposed research, with extensive infrastructure for both plant physiology and molecular biology experiments. The lab has adequate space and facilities to accommodate all planned experiments, including the integration of new equipment. As a recently selected Zuckerman Faculty Scholar, I have secured an additional $500,000 in funding, strengthening our capacity to support lab personnel and expand research capabilities. This substantial funding ensures resources for both equipment acquisition and the expansion of our research program. The proposal implementation section has been updated to reflect these enhanced capabilities.

**Response to reviewer #3:**

1. Na+ sinks in tomato fruit under salt stress: Thank you for this insightful comment. In Objective 3, my goal is to assess the functionality of candidate genes involved in rootward Na⁺ recycling using transiently transfected plants. Studies by Raveh & Ben-Gal (2016) and Roșca *et al*., (2023) suggest that under salt stress, Na⁺ concentration can moderately increase in tomato fruits, suggesting that fruits may act as partial Na⁺ sinks. While the reviewer’s suggestion to investigate fruits as potential Na⁺ unloading sites is both relevant and aligns with my interests, accurately evaluating this role under prolonged salt stress would require generating stably transgenic or mutant plants- a process that exceeds this proposal’s five-year timeframe. To meet the objectives within the current timeline and provide a foundation for future studies on Na⁺ dynamics and their impact on growth and fruit quality, Objective 3 will focus on investigating alternative Na⁺ sinks, such as the petiole, stem, or roots, in transiently transfected plants.

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